# Ghrelin attenuates hepatocellular injury and liver fibrogenesis in rodents and influences fibrosis progression in humans

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**Abbreviations:** GHS-R, growth hormone secretagogue receptor; *Ghrl*$^{-/-}$, ghrelin knockout; $CCl_4$, carbon tetrachloride; *Ghrl*$^{+/+}$, wild type; BDL, bile duct ligation; HSC, hepatic stellate cells; TUNEL, Terminal dUTP Nick End Labeling; *GHRL*, ghrelin gene.

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**There are no effective antifibrotic therapies for patients with liver diseases. We performed an experimental and translational study to investigate whether ghrelin, an orexigenic hormone with pleiotropic properties, modulates liver fibrogenesis. Recombinant ghrelin was administered to rats with chronic (bile duct ligation) and acute (carbon tetrachloride) liver injury. Hepatic gene expression was analyzed by microarray analysis and quantitative polymerase chain reaction. The hepatic response to chronic injury was also evaluated in wild type and ghrelin-deficient mice. Primary human hepatic stellate cells were used to study the effects of ghrelin *in vitro*. Ghrelin hepatic gene expression and serum levels were assessed in patients with chronic liver diseases. Ghrelin gene polymorphisms were analyzed in patients with chronic hepatitis C. Recombinant ghrelin treatment reduced the fibrogenic response, decreased liver injury, myofibroblast accumulation and attenuated the altered gene expression profile in bile duct-ligated rats. Moreover, ghrelin reduced the fibrogenic properties of hepatic stellate cells. Ghrelin also protected rats from acute liver injury and reduced the extent of oxidative stress and inflammation. Ghrelin-deficient mice, developed exacerbated hepatic fibrosis and liver damage after chronic injury. In patients with chronic liver diseases, ghrelin serum levels decreased in those with advanced fibrosis and ghrelin gene hepatic expression correlated with expression of fibrogenic genes. In patients with chronic hepatitis C, polymorphisms of the ghrelin gene (–994CT and –604GA) influenced the progression of liver fibrosis. *Conclusion:* Ghrelin exerts antifibrotic effects in the liver and may represent a novel antifibrotic therapy.**

Hepatic fibrosis is the progressive accumulation of extracellular matrix that occurs in most types of chronic liver diseases. In patients with advanced fibrosis, liver cirrhosis ultimately develops. Currently, the only effective therapy to treat liver fibrosis is to eliminate the causative agent (e.g. successful antiviral therapy in patients with chronic hepatitis C). For those patients in whom the underlying cause cannot be removed, there are no effective antifibrotic therapies. During the last years, research has focused on molecular and cellular mechanisms involved in liver fibrosis and many pharmacological interventions have been successfully tested in experimental models of liver fibrosis.(1) However, most of the information derives from the experimental setting, while translational studies with human samples and clinical trials are scarce. In the current study, we used both experimental and translational approaches to characterize a new potential antifibrotic substance for patients with chronic liver diseases.

Ghrelin is a gut hormone (28-amino-acids) firstly discovered as a potent growth hormone secretagogue. Moreover, plays a major role in the regulation of food intake.(2) Recently, peripheral effects such as cytoprotection, vasodilatation, regulation of energy balance and gastrokinesis have been also attributed to ghrelin.(3) The primary site of ghrelin synthesis is the stomach but ghrelin transcripts have been detected in many other organs including the liver (e.g. bowel, pancreas, kidneys, lungs, etc).(4) Most of ghrelin actions are mediated by Growth Hormone Secretagogue Receptor (GHS-R),(2) which is mainly expressed in the pituitary gland but also in other organs, including pancreas, spleen and adrenal gland.(4) However, ghrelin probably binds to another yet unknown receptor because cells not expressing GHS-R respond to ghrelin stimulus.(5)

Recent data indicate that ghrelin has protective effects in different organs and cell types including the pancreas, the heart and the gastrointestinal tract.(6-8) Recombinant ghrelin has been successful administered to patients with a variety of disorders such as anorexia,(9) caquexia(10) and gastroparesis(11). Moreover, ghrelin reduces muscle wasting and

improves functional capacity in elderly patients with congestive heart failure and chronic obstructive pulmonary disease.(12, 13) We hypothesize that ghrelin regulates hepatic injury and fibrogenesis. To prove this hypothesis, we investigated the effect of recombinant ghrelin in different models of acute and chronic liver injury. Moreover, we evaluated whether changes in endogenous ghrelin regulate hepatic fibrosis in mice and in patients with chronic liver diseases due to hepatitis C virus infection. We provide evidence that recombinant ghrelin exerts protective and antifibrotic effects in the injured liver. Our results also suggest that endogenous ghrelin plays a role in hepatic fibrogenesis since ghrelin knockout (*Ghrl*$^{-/-}$) mice are more susceptible to carbon tetrachloride ($CCl_4$) induced liver injury than wild type (*Ghrl*$^{+/+}$) mice. Moreover, we demonstrate that ghrelin is locally produced in the human liver.

## Materials and Methods

***Chronic liver injury models in rodents.*** Male Wistar rats (250 g) were induced to chronic liver injury and hepatic fibrosis by prolonged bile duct ligation (BDL), as described previously.(14) Either saline, rat recombinant ghrelin (Phoenix Pharmaceuticals; Burlingame, CA) or ghrelin receptor agonist (Des-Ala$^3$-GHRP-2) (Bachem; Bubendorf, Switzerland) were administered into rats through a subcutaneous osmotic minipump (Alza Corporation; Palo Alto, CA) at a rate of 200 $\mu l \cdot h^{-1}$ throughout the experiment. Doses were chosen from existing data in the literature. Preliminary studies in rats with advanced fibrosis ($CCl_4$ for 8 weeks) were performed to assess the tolerability of both ghrelin and (Des-Ala$^3$-GHRP-2). The selected doses for the peptides (10 $\mu g \cdot Kg^{-1} \cdot d^{-1}$ for recombinant ghrelin and 30 $\mu g \cdot Kg^{-1} \cdot d^{-1}$ for Des-Ala$^3$-GHRP-2) were well tolerated and did not cause arterial hypotension. Experimental groups were as follows (n=12 per group): rats with BDL or sham-operated rats and infused with saline, recombinant rat ghrelin or the ghrelin receptor agonist (Des-Ala$^3$-GHRP-2). *Ghrl*$^{-/-}$ mice (C57BL/6 background) were obtained from Regeneron Pharmaceuticals (Tarrytown, NY). The generation and characterization of these mice has been extensively described previously.(15) We used mice aged 8–10 weeks. Because C57BL/6 mice early develop biliary infarcts and have a high rate of mortality following bile duct ligation,(16) we used a different experimental model to induce chronic liver injury and hepatic fibrosis. Carbon tetrachloride ($CCl_4$) (Sigma-Aldrich; St. Louis, MO) was administered intraperitoneally at a dose of 1 $mL \cdot Kg^{-1}$, 12.5% diluted in olive oil (Sigma-Aldrich) twice a week during 4 weeks. Control mice were administered olive oil at the same dose. Each group included at least 12 mice. Rats and mice were housed in temperature and humidity-controlled rooms and kept on a 12-h light cycle. Animal procedures were approved by the Ethics Committee of Animal Experimentation of the Universitat de Barcelona and were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health.

***Assessment of hepatic necroinflammatory injury and fibrosis.*** Paraffin-embedded liver sections were stained with H&E. Hepatic necroinflammation was estimated by quantifying the presence of necrosis, hepatocyte ballooning and/or swelling, inflammatory cell infiltration and lipid droplets. The degree of necroinflammatory changes was assessed as the percentage of hepatic parenchyma with any of the above described changes: 1, lower than 30%; 2, 30–60%; 3, more than 60%. Analyses were blindly performed by an expert pathologist (LNZR). To assess liver fibrosis, liver specimens were stained with picrosirius red (Gurr-BDH Lab Supplies; Poole, England). The positive area stained with picrosirius red was quantified using a morphometric method. Briefly, six images per specimen were obtained with an optic microscope (Nikon Corporation; Tokyo, Japan) at magnification of 40x. Images were imported to an image-analysis software (AnalySIS, Olympus; Münster, Germany) and automatically merged.

***Acute liver injury model in rats.*** Acute liver injury was induced in male Wistar rats (250 g) by a single intraperitoneal injection of $CCl_4$ (Sigma-Aldrich; 1 $mL \cdot Kg^{-1}$ body weight, 30% diluted in olive oil). Control rats received the same amount of olive oil. Animals were treated with either saline or 20 $\mu g \cdot Kg^{-1}$ rat recombinant ghrelin (Phoenix Pharmaceuticals) intravenously one hour before $CCl_4$ administration. Rats were divided into three experimental groups (n=8 per group): rats receiving saline and olive oil, rats receiving saline and $CCl_4$ and rats receiving ghrelin and $CCl_4$. Twenty-four hours after the intraperitoneal injection, animals were anesthetized and sacrificed for blood and tissue samples collection. Rats were housed in temperature and humidity-controlled rooms and kept on a 12-h light cycle. Animal procedures were conducted in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health.

***Human samples.*** For analysis of ghrelin serum levels, blood samples from patients with chronic hepatitis C (n=67) and alcoholic hepatitis (n=24) were obtained. Moreover, samples from healthy controls (n=24) matched for age, gender and body mass index with

patients were collected. Blood samples were obtained after an overnight fasting. Hepatic gene expression was assessed in liver specimens obtained by a transjugular approach from patients with alcoholic hepatitis (n=37) and by a percutaneous approach in patients with chronic hepatitis C (n=45) and in patients with non alcoholic steatohepatitis (n=23). Normal liver specimens (n=5) were obtained from fragments of resections of colon metastases before the vascular clamping as described previously.(17) For the analysis of the role of variations of the ghrelin gene on the progression of liver fibrosis, DNA from patients with chronic hepatitis C (n=284) was obtained from peripheral blood. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the Hospital Clínic of Barcelona. All patients gave informed consent.

***Data analysis.*** Data are representative of at least three independent experiments. Results are expressed as mean ± SEM. The normality of the data was assessed by the Kolmogorov-Smirnov test. Comparisons between groups were performed by Student t-test or non parametric Mann-Whitney test depending on the normality of data. Statistical analysis of correlations was performed by Spearman rho. *P* values <0 .05 were considered significant. For multiple comparisons, Bonferroni correction was applied to *P* values, with significance set at $P<0.001$.

Other methods are shown in Supplementary Materials and Methods.

## Results

***Liver fibrosis is reduced in rats treated with recombinant ghrelin.*** To investigate whether recombinant ghrelin regulates hepatic fibrogenesis following chronic liver injury, a model of secondary biliary fibrosis was induced in rats through prolonged ligation of the common bile duct. Both bile duct ligated or sham operated rats were continuously infused with either saline or recombinant ghrelin through a subcutaneous osmotic pump for 2 weeks. Bile duct ligated rats infused with saline showed severe septal hepatic fibrosis with a marked disruption of the hepatic architecture (Fig. 1A). Hepatic collagen content was increased over 7-fold compared to control rats. In contrast, bile duct ligated rats infused with ghrelin had only mild collagen deposition without formation of bridging fibrosis. Morphometric analysis revealed that ghrelin decreased collagen deposition about 40%. To uncover the mechanisms underlying this beneficial effect, we first investigated whether ghrelin modulates the accumulation of myofibroblastic fibrogenic cells (smooth muscle $\alpha$-actin positive cells). Myofibroblastic cells markedly accumulated throughout the hepatic parenchyma in bile duct ligated rats. Ghrelin treatment reduced the amount of fibrogenic cells by 25% (Fig. 1B). Moreover, ghrelin treatment decreased $\alpha$-SMA protein expression, as assessed by western blot (Fig. 1C) and hepatic content of hydroxyproline (Fig. 1D). In addition, ghrelin infusion reduced the elevation of serum aspartate aminotransferase levels, a parameter indicative of hepatocellular damage, induced by bile duct ligation (Fig. 1E). Because ghrelin stimulates guanosin 3',5'-cyclic monophosphate production in other tissues(18) we next studied whether the beneficial effect of ghrelin is associated with increased guanosin 3',5'-cyclic monophosphate hepatic content. We did not find differences between any of the groups (Fig. 1F).

***Recombinant ghrelin prevents changes in hepatic gene expression during liver fibrogenesis.*** To explore the effects induced by ghrelin in the fibrotic liver, we analyzed changes in hepatic gene expression by complementary DNA microarray analysis. Bile duct ligation stimulated the hepatic expression of 1543 genes and repressed the expression of

997 genes compared to sham-operated rats. Ghrelin treatment attenuated changes in the expression of 231 genes including collagen-α1(II), plasminogen activator-urokinase receptor, matrix metallopeptidase 2 and chemokine receptor 5 (Fig. 2A). A list of all the genes modified by ghrelin treatment is shown in Supplementary Table 1. The complete dataset is available at the NCBI's Gene Expression Omnibus public database (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE13747. Quantitative polymerase chain reaction confirmed the changes found in microarray analysis in some selected genes (Fig. 2B). Rat liver samples were clusterized depending on gene expression profile. Rats were perfectly classified in the different experimental groups. A heatmap of the clustering can be seen in Supplementary Fig. 1.

***Increased liver injury and fibrogenesis in Ghrl$^{-/-}$ mice.*** To investigate the role of endogenous ghrelin in liver fibrogenesis, we next analyzed the fibrogenic response in *Ghrl*$^{-/-}$ and *Ghrl*$^{+/+}$ mice. Chronic liver injury was induced by intraperitoneal injections of $CCl_4$ twice a week for 4 weeks. The extent of liver fibrosis was assessed in both groups of mice. We found that ghrelin deficient mice were more susceptible to $CCl_4$-induced liver fibrosis and liver injury than wild type mice, as indicated by increased collagen deposition (Fig. 3A and B) and increased necroinflammatory score (Fig. 3C). Moreover, *Ghrl*$^{-/-}$ mice treated with $CCl_4$ showed a reduced weight gain compared to *Ghrl*$^{+/+}$ mice (Fig. 3D). In addition, procollagen-α2(I) and TIMP1 expression were overexpressed in ghrelin deficient mice treated with $CCl_4$ compared to wild type littermates (Fig. 3E and 3F).

***A GHS-R agonist attenuates liver fibrosis.*** We first analyzed by polymerase chain reaction the expression of GHS-R in human and rat liver samples. We found transcripts of GHS-R in both human and rat livers (Fig. 4A and B). Specifically, we detected GHS-R expression in human hepatocyes and activated hepatic stellate cells (HSC) but not in quiescent HSC (Figure 4B). To investigate whether stimulation of GHS-R attenuates liver fibrosis new groups of rats were submitted to BDL or sham operation in the presence or

absence of a GHS-R agonist (Des-Ala$^3$-GHRP-2) for two weeks. We found that the degree of liver fibrosis was reduced in rats treated with the GHS-R agonist, as indicated by decreased collagen deposition (Fig. 4C and D).

***Recombinant ghrelin reduces hepatocellular injury in a model of acute liver injury in rats.*** The results in bile duct-ligated rats suggest that ghrelin may attenuate fibrosis by exerting a hepatoprotective effect. To prove this hypothesis, we analyzed the effects of ghrelin in a model of acute liver injury in rats (single intraperitoneal administration of $CCl_4$). Ghrelin or vehicle were administered to rats intravenously one hour before $CCl_4$. Pre-treatment with ghrelin, but not saline, strongly reduced the hepatocellular injury induced by $CCl_4$, as indicated by decreased necroinflammatory score (Fig. 5A) and aspartate aminotransferase serum levels (170 and 90 IU/l in $CCl_4$-damaged rats in the absence and the presence of ghrelin respectively, $P<0.05$). This beneficial effect was associated with decreased infiltration of inflammatory cells, as assessed by quantification of infiltrating leucocytes (CD43-positive cells) in liver sections ($P<0.05$, Fig. 5B). Because oxidative stress mediates $CCl_4$-induced hepatocellular injury, we also explored whether ghrelin reduces this pathogenic event by quantifiying 4-hydroxynonenal protein adducts. As shown in Fig. 5C, ghrelin attenuated the accumulation of 4-hydroxynonenal in hepatocytes. We next explored the effects on hepatocyte cell death by Terminal dUTP Nick End Labeling (TUNEL) analysis. Ghrelin diminished the number of TUNEL-positive hepatocytes, indicating that it reduces cell apoptosis (Fig. 5D). This effect was associated with decreased activation of nuclear factor κB, as assessed by p65 nuclear translocation (Fig. 5E). Moreover, ghrelin treatment attenuated the effects of $CCl_4$ on Akt and extracellular signal-regulated kinase ERK phosphorylation, two intracellular pathways involved in hepatocyte survival and proliferation (Fig. 5F). All together, these results indicate that ghrelin exerts hepatoprotective effects.

***Ghrelin modulates fibrogenic, but not proinflammatory, properties of hepatic stellate cells.*** To further elucidate possible mechanisms of the protective effects of ghrelin in the liver, we next investigated whether ghrelin modulates the fibrogenic actions of HSC, the main fibrogenic cell type in the injured liver.(1) Stimulation of primary cultured HSC with angiotensin II (0.1 μM), a well-known fibrogenic agonist, resulted in a marked increase in intracellular calcium concentration ($[Ca^{2+}]_i$). Pre-incubation with ghrelin (0.1 μM) for 10 minutes attenuated angiotensin-II induced $[Ca^{2+}]_i$ increase (Fig. 6A). Ghrelin (0.1 μM) also reduced by 40% the expression of collagen-α1(I) and transforming growth factor-β1 in unstimulated HSC (Fig. 6B). We then investigated whether ghrelin inhibits the pro-inflammatory actions of HSC. Ghrelin did not modulate the activation of nuclear factor κB or the release of interleukin-8 (Fig. 6C and D respectively). These results indicate that ghrelin reduces the fibrogenic but not the inflammatory properties of cultured HSC.

***Serum ghrelin levels and hepatic ghrelin expression in patients with chronic liver diseases.*** To analyze the potential role of ghrelin in chronic human liver diseases, serum ghrelin concentration was measured in control subjects (n=24) and in patients with liver fibrosis including alcoholic hepatitis (n=24) and chronic hepatitis C (n=67). Serum ghrelin levels were significantly lower in both patients with alcoholic hepatitis and chronic hepatitis C compared to control subjects, after adjusting by age, gender and body mass index (Fig. 7A). Interestingly, ghrelin serum levels were lower in patients with advanced fibrosis (Metavir score 3–4) than in those with mild fibrosis (Metavir score 0–2) (Fig. 7B). Next, we assessed ghrelin gene (*GHRL)* expression in normal (n=5) and diseased human livers (37 patients with alcoholic hepatitis, 45 patients with chronic hepatitis C and 23 patients with non alcoholic steatohepatitis). Ghrelin transcripts were found in both normal and diseased livers. Interestingly, *GHRL* was clearly overexpressed in livers with non alcoholic steatohepatitis compared to the rest of groups (Fig. 7C). Moreover, in the whole series of patients with chronic liver diseases, *GHRL* hepatic expression positively correlated with the expression of genes involved in fibrogenesis (Supplementary Table 3) as well with BMI

(r=0.675, $P < 0.0001$). At the cellular level, *GHRL* transcripts were found in both hepatocytes and HSC freshly isolated from human livers as well as in culture-activated human HSC (Fig. 7D).

***Polymorphisms in the ghrelin gene are associated with the degree of fibrosis in patients with chronic hepatitis C.*** Finally, we investigated whether ghrelin gene polymorphisms are associated with the progression of liver fibrosis in patients with chronic liver diseases. For this purpose, we analyzed six single nucleotide polymorphisms on the ghrelin gene (Supplementary Fig. 2A): –994CT, –604GA, –501AC, Arg51Gln, Met72Leu and Leu90Gln (GeneBank numbers can be found in Supplementary Materials and Methods) in 284 patients with HCV-induced liver disease. One single nucleotide polymorphisms in the promoter (–994CT) was differently represented between women with advanced fibrosis (F3-F4) and those with mild fibrosis (F0-F2). Moreover, we found that patients with the haplotype –994T and –604A are more susceptible to severe liver fibrosis after adjusting by age and gender (Table 1). These results suggest that variations in *GHRL* modulate the progression of chronic hepatitis C. To investigate the functionality of these polymorphisms, we constructed plasmids containing the promoter of ghrelin with different haplotypes (wild type and–994CT –604GA) bound to the luciferase gene. Plasmids were transfected to Huh7 hepatocytes. The plasmid with the promoter containing the haplotype associated with an increased risk to develop advanced fibrosis was found to be more active than the plasmid containing the wild type promoter (Supplementary Fig. 2B).

## Discussion

Gut hormones play a major role in food intake and energy homeostasis at different levels, from central regulation of appetite to motility of the gastrointestinal tract. They also regulate inflammatory and fibrogenic processes in a variety of tissues. Ghrelin is a gut hormone that is also produced by extraintestinal tissues and exerts a variety of pleiotropic effects in parenchymal cells.(3) We provide extensive evidence that ghrelin exerts antifibrotic and hepatoprotective effects in the injured liver in rodents. We demonstrate that recombinant ghrelin regulates the fibrogenic response of the liver to acute and chronic injury. Moreover, endogenously-produced ghrelin also regulate fibrogenesis in mice and humans. The hepatoprotective effects of ghrelin confirm previous studies indicating that ghrelin exerts protective effects in parenchymal cells and in damaged tissues such as the heart and the colon.(6, 19) In the liver, a single study(20) suggests protective effects of ghrelin in a model of chronic liver injury. Our study extensively expands this notion by demonstrating a role for ghrelin in liver fibrosis. This new effect of ghrelin has potential therapeutic implications, as discussed later.

The main finding of our study is that ghrelin regulates hepatic fibrosis. Although a number of studies have suggested that ghrelin has protective effects against cell death,(5, 21) the current study expands this effect by demonstrating that ghrelin also prevents scar tissue formation in chronically injured tissues. Most importantly, we demonstrate for the first time that endogenously produced ghrelin regulates fibrogenesis in the liver. Besides the effects in experimental models of liver injury (BDL and $CCl_4$), we used a translational approach to study the potential role of ghrelin in samples from patients with chronic liver injury. First, we analyzed ghrelin hepatic expression in patients with different liver diseases. We found ghrelin expression in both normal and diseased livers. Interestingly, obesity and the presence of non alcoholic steatohepatitis were associated with increased hepatic expression of ghrelin. This interesting result is probably related to the deregulated energetic metabolism in obese subjects and deserves further investigation. We also analyzed serum ghrelin levels in

patients with chronic liver diseases. We found that ghrelin serum levels decreased in patients with advanced fibrosis. Our results apparently differ from a recent report showing that ghrelin serum levels are increased in patients with chronic liver diseases.(22) In this latter study, ghrelin serum levels were increased in patients with advanced cirrhosis. This advanced state is associated with profound hepatic failure, caquexia, endotoxinemia and hemodynamic disturbances, which could influence serum levels of cytokines and vasoactive substances. In our series, the vast majority of patients have mild to moderate degree of fibrosis, which could explain the discrepant results. Finally, we studied the role of ghrelin gene variations in the progression of liver fibrosis, in a well-characterized series of patients with biopsy-proven chronic hepatitis C. We analyzed *GHRL* polymorphisms and compared their frequencies in patients with mild fibrosis and patients with advanced fibrosis. We found two single nucleotide polymorphisms in the *GHRL* associated with advanced fibrosis in women but not in men. The fact that polymorphisms affect mainly women is a very intriguing question. It is well known that gender is a major factor influencing ghrelin expression and serum levels.(23, 24) In fact, previous studies indicate that gender markedly influences the effect of ghrelin polymorphisms in different diseases.(25, 26) Therefore, it is not surprising that in our study the influence of ghrelin polymorphisms on liver fibrosis were gender-dependent. Further studies are required to investigate this issue. Moreover, it is well known that fibrosis progression is modulated by estrogens.(27)

Different mechanisms may explain the antifibrotic effects of ghrelin in the injured liver. First, ghrelin seems to protect hepatocytes from cell death, as indicated by decreased necroinflammatory injury and serum levels of aminotransferases in rats subjected to both acute and chronic liver injury. This effect was related to a reduction in the number of infiltrating inflammatory cells as well as decreased apoptosis in hepatocytes in the model of acute liver injury. These results confirm previously published data indicating that ghrelin prevents parenchymal cell death in different injured tissues.(8, 18, 28) Interestingly, we found that ghrelin administration to injured rats resulted in increased hepatic expression of

hepatoprotective signaling pathways such as phospho-Akt and phospho- extracellular signal-regulated kinase. These results are in keeping with several studies showing that ghrelin induces activation of Akt and extracellular signal-regulated kinase in different cell types.(5, 7, 29) Second, we found that ghrelin decreases the extent of oxidative stress in the liver, which is a major pathogenic event in the wound healing response to injury. This antioxidant effect of ghrelin has been shown in other organs.(30, 31) Whether ghrelin reduces the formation of reactive oxygen species or increases the activity of antioxidant defenses is unknown and deserves further investigation. Fourth, we provide evidence that ghrelin reduces the accumulation of activated HSC in the liver and it directly reduces collagen synthesis by cultured HSC. This effect is associated with decreased TGF-β1 expression, a major profibrogenic cytokine in the liver. Finally, microarray analysis revealed several potential mechanisms by which ghrelin could exert its antifibrotic effect. Thus, besides reducing expression of genes involved in extracellular matrix synthesis, ghrelin reduced the expression of genes involved in apoptosis (caspases), inflammation (osteopontin, chemokine receptor 5) and cellular contractility (tropomyosin).

This study has several limitations. First, it is unknown whether locally produced ghrelin or extrahepatic synthesis of ghrelin (e.g. by the stomach) regulate hepatic fibrogenesis. The finding that ghrelin serum levels are decreased in patients with more aggressive fibrosis suggests that extrahepatic sources of ghrelin could be implicated in the progression of fibrosis. Second, further studies using GHS-R antagonists should confirm the involvement of this receptor in the beneficial effects induced by ghrelin. Third, the role of ghrelin in fibrosis resolution and the therapeutic effect of exogenous ghrelin in established cirrhosis should be evaluated. Fourth, since ghrelin requires a post-traslational modification (octanylation) to be active,(32) further analysis of the ghrelin active form should be performed in liver samples and cell types. Fifth, the results in *Ghrl*$^{-/-}$ are less impressive than in rats receiving recombinant ghrelin probably because constitutive knockout mice usually develop strategies to overcome the lack of a given gene. Further studies using ghrelin conditional knockout

mice and/or ghrelin receptor knockout mice are should clarify this question. And sixth, although we provide evidence that ghrelin exerts direct antifibrotic effects in fibrogenic cells, the precise molecular mechanisms by which ghrelin exerts beneficial effects in liver undergoing acute and/or chronic injury should be uncovered in further studies.

The results of our study have potential therapeutic implications. Recombinant ghrelin has been tested in patients with different conditions including gastroparesis(11) anorexia(9), caquexia(10) and chronic heart failure.(12) In these studies, ghrelin is generally well tolerated and only causes a mild decrease in arterial pressure. Our results suggest that ghrelin could also be useful in patients with liver injury and liver fibrosis. Further studies should evaluate this hypothesis. Moreover, due to the orexigenic properties of ghrelin, ghrelin receptor antagonists have been recently proposed for the treatment of diabetes and obesity.(33) Due to its protective effects, prolonged blockade of ghrelin receptors may cause adverse effects such as accelerated tissue fibrosis, which is commonly seen in the heart and the kidney of patients with metabolic syndrome.

In summary, the results of the current study indicate that ghrelin exerts hepatoprotective and antifibrogenic effects in the liver. Further studies should evaluate the safety and efficacy of ghrelin and/or ghrelin agonists in patients with chronic liver diseases.

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**Figure 1.** Ghrelin reduces hepatic fibrosis induced by bile duct ligation (BDL) in rats. (A) Representative pictures of Sirius red staining (original magnification, x40) from rats submitted to BDL or sham-operated rats treated with saline or ghrelin. Graph shows quantification of Sirius red stained area. (B) Representative pictures and quantification of smooth muscle α-actin positive cells, α-SMA (original magnification, x400). (C) Representative western blot for α-SMA in liver samples. (D) Hydroxyproline content in liver samples from sham-operated rats or rats submitted to bile duct ligation treated or not with ghrelin (E) Aspartate aminostransferase serum levels from all groups of rats. (F) Guanosin 3',5'-cyclic monophosphate hepatic content in liver extracts from all groups of rats. Data shown are mean from at least 10 animals per group; error bars show SEM. $^{\#}P < 0.05$ respect to sham-operated rats, $^{*}P < 0.05$ respect to saline-BDL rats.

**Figure 2.** Hepatic gene expression in rats submitted to sham-operation or bile duct ligation (BDL). (A) Microarray data from hepatic complementary DNA. Expression of key genes was modified by BDL. Ghrelin treatment attenuated changes in gene expression profile. All genes have a False Discovery Rate < 0.2 and are deviated from the control by at least ± 1.8 fold (B) Quantitative polymerase chain reaction confirmed the results obtained in the microarray analysis in procollagen-α1(II) (*Col1a2*), matrix metallopeptidase 2 (*Mmp2*), endothelin receptor type A (*Ednra*) and sterol regulatory element binding factor 1 (*Srebf1*). Data shown are mean from at least 5 animals per group; error bars show SEM. $^{\#}P < 0.05$ respect to sham-operated rats, $^{*}P < 0.05$ respect to saline-BDL rats.

**Figure 3.** Role of endogenous ghrelin in liver fibrosis in mice. *Ghrl*$^{+/+}$ and *Ghrl*$^{-/-}$ mice were induced to liver fibrosis by administration of $CCl_4$ for 4 weeks. *Ghrl*$^{-/-}$ mice show a modest increase in the extent of liver fibrosis and increased liver damage after chronic liver injury induced by $CCl_4$ compared to *Ghrl*$^{+/+}$. (A) Representative pictures and (B) quantification of Sirius red staining (original magnification, x40) from *Ghrl*$^{+/+}$ and *Ghrl*$^{-/-}$ mice treated

chronically with oil or $CCl_4$. (C) Necroinflammatory score of liver samples from *Ghrl*$^{+/+}$ and *Ghrl*$^{-/-}$ mice chronically treated with oil or $CCl_4$. (D) Weight increase during the four weeks of $CCl_4$ treatment in all groups of mice. (E) and (F) Gene expression of genes involved in fibrogenesis. Procollagen-α2(I) and tissue inhibitor of metalloproteases (TIMP-1) were overexpressed in ghrelin deficient mice induced to liver fibrosis when compared with wild type mice. Data shown are mean from at least 10 animals per group; error bars show SEM. $^{\#}P < 0.05$ respect to oil-treated mice, $^{*}P < 0.05$ respect to $CCl_4$-wild type mice.

**Figure 4.** Effects of a GHS-R agonist, (Des-Ala3)-GHRP-2, on experimental liver fibrosis. Expression of GHS-R was detected in rat (A) and human (B) livers. NC, negative control; PC, positive control; L, liver; Hep, hepatocytes; Q-HSC, quiescent HSC and A-HSC, activated HSC. A GHS-R agonist, (Des-Ala3)-GHRP-2, was infused in sham-operated rats and rats with BDL during the two weeks of the experiment. (C) Representative pictures and (D) quantification of the area stained by Sirius red (original magnification, x40). Data shown are mean ± SEM from 8 rats per group. $^{\#}P < 0.05$ respect to sham, $^{*}P < 0.05$ respect to saline-BDL.

**Figure 5.** Ghrelin exerts hepatoprotective effects in rats with acute liver injury induced by $CCl_4$. Rats received ghrelin (20 μg·Kg-1) intravenously one hour before $CCl_4$ administration. (A) Representative pictures of H&E staining in livers from $CCl_4$-injured rats treated with saline or ghrelin (original magnification, x200). Carbon tetrachloride induced hepatocyte ballooning, parenchymal necrosis and inflammatory infiltrate. Graph shows evaluation of the necroinflammatory score. (B) Representative pictures of CD43 immunostaining in $CCl_4$-treated rats (original magnification, x400). Graph shows quantification of CD43-positive cells per field (x200 magnification). (C) Representative pictures of 4-hydroxynonenal immunostaining in $CCl_4$-treated rats (original magnification, x400). Quantification of the area stained is shown in the graph. (D) Representative pictures of TUNEL immunostaining in $CCl_4$-treated rats (original magnification, x400). Graph shows quantification of TUNEL-

positive cells per field (x400 quantification). (E) Representative pictures of p65 immunostaining (original magnification, x400). Graph shows quantification of p65-positive nuclei per field (x400 magnification). $^{\#}P < 0.05$ respect to control, $^{*}P < 0.05$ respect to rats receiving saline-$CCl_4$. (F) Intracellular pathways involved in $CCl_4$-induced liver damage and ghrelin hepatoprotection. Western blot studies showing Akt and extracellular signal-regulated kinase phosphorylation in extracts from rat livers. Numbers underneath represent fold expression compared with oil-treated rats. Data shown are mean ± SEM from 8 animals per group.

**Figure 6.** Effects of ghrelin on pro-fibrogenic and proinflammatory properties in primary human HSC. (A) Intracellular calcium concentration ($[Ca^{2+}]$) as evidenced by Fura 2 intensity in HSC. Cells were pre-incubated for 10 minutes with ghrelin (0.1 μmol/L) and then challenged with angiotensin II (0.1 μmol/L). (B) Expression of procollagen-α1(I) and TGF-β1 mRNA in HSC exposed to ghrelin (0.1 μmol/L) for 24 hours. $^{*}P < 0.05$ respect to vehicle. (C) Activity of nuclear factor κB assessed by luciferease reporter gene assay. Cells were infected with an adenovirus containing luciferase gene with the promoter region for nuclear factor κB transcription factor and incubated overnight with vehicle, ghrelin or phorbol 12-myristate 13-acetate (PMA). Ghrelin (0.1 μmol/L) did not modulate nuclear factor κB activity in HSC. 12-myristate 13-acetate (1mg/mL) was used as a positive control. Preincubation of cells with ghrelin for 10 minutes did not modulate the effect of 12-myristate 13-acetate. (D) Cells were incubated with vehicle, ghrelin or tumor necrosis factor-α (TNF-α) for 24 hours. Medium was collected to analyze interleukin 8 concentration. Ghrelin (0.1 μmol/L) did not modulate interleukin 8 release by HSC to the culture medium. Tumor necrosis factor α (1 ng/mL) was used as a positive control. Preincubation of cells with ghrelin for 10 minutes did not modulate the effect of TNF-α;. Data shown are mean ± SEM from 3 independent experiments.

**Figure 7.** Ghrelin serum levels and hepatic ghrelin expression in control subjects and in patients with chronic liver diseases. (A) Fasting ghrelin serum levels were analyzed in blood samples from patients with chronic HCV infection, alcoholic hepatitis (AH) and healthy controls. Serum ghrelin levels were decreased in all groups of patients. (B) Ghrelin levels were lower in patients with advanced fibrosis compared to those with mild fibrosis $^{*}P < 0.05$ respect to control or to F0-F2. (C) *GHRL* hepatic expression was analyzed in samples from controls, chronic hepatitis C, AH and non alcoholic steatohepatitis (NASH) patients $^{*}P < 0.05$ respect to all the groups. (D) Ghrelin expression was analyzed in different hepatic cell types. NC, negative control; Hep, primary human hepatocytes; Q-HSC, quiescent human hepatic stellate cells; A-HSC, human in culture-activated HSC.

**Table 1.** Effects of ghrelin genetic polymorphisms in the progress of fibrosis in patients with chronic hepatitis C.

**Supplementary Figure 1.** Heatmap showing clustering in rat liver samples according to similarity in gene expression. Rats were classified according to hepatic gene expression profile. C, sham-saline; t1, BDL-ghrelin; t0, BDL-saline.

**Supplementary Figure 2.** (A) Gene structure of the ghrelin gene showing the SNPs analyzed. Six ghrelin gene SNPs were analyzed in patients with chronic hepatitis. C. Three of them were located on the promoter, two of them in introns and one of them was in the codon of the last aminoacid of the mature protein. (B) Analysis of ghrelin promoter activity. Plasmids with different haplotypes for the ghrelin promoter linked to the luciferase gene were transfected to Huh7 cells. Ghrelin promoter activity was assessed by luminiscence. $^{*}P < 0.05$ respect to the control vector. $^{\#}P < 0.05$ respect to wild type haplotype.

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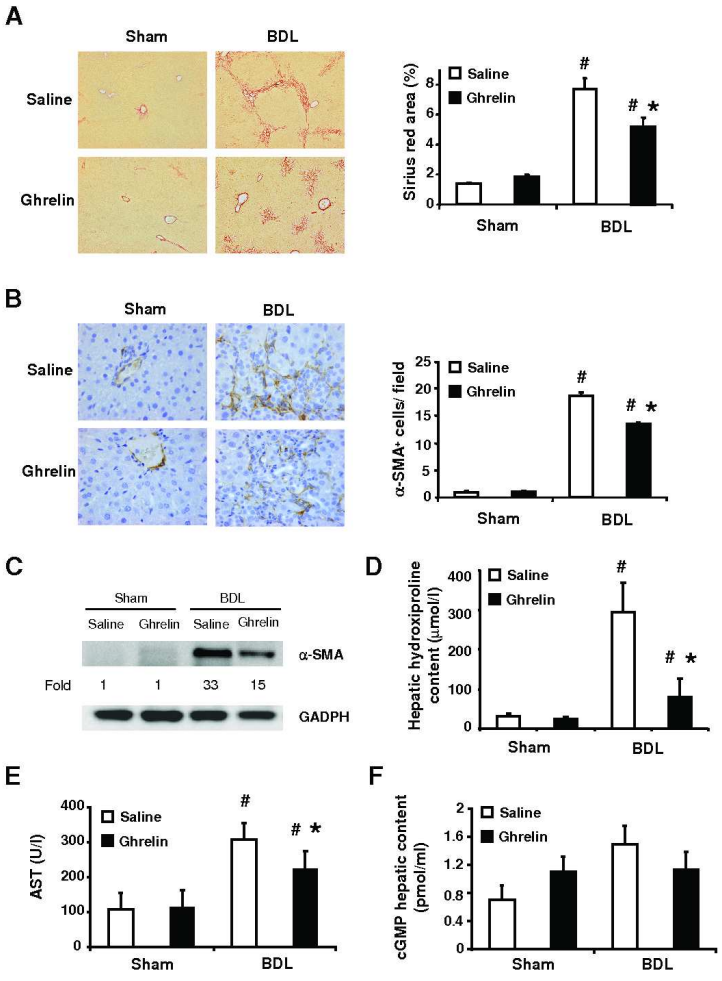


Figure 1. Ghrelin reduces hepatic fibrosis induced by bile duct ligation (BDL) in rats. (A) Representative pictures of Sirius red staining (original magnification, x40) from rats submitted to BDL or sham-operated rats treated with saline or ghrelin. Graph shows quantification of Sirius red stained area. (B) Representative pictures and quantification of smooth muscle α-actin positive cells, α-SMA (original magnification, x400). (C) Representative western blot for α-SMA in liver samples. (D) Hydroxyproline content in liver samples from sham-operated rats or rats submitted to bile duct ligation treated or not with ghrelin (E) Aspartate aminostransferase serum levels from all groups of rats. (F) Guanosin 3',5'-cyclic monophosphate hepatic content in liver extracts from all groups of rats. Data shown are mean from at least 10 animals per group; error bars show SEM. #P < 0.05 respect to sham-operated rats, *P < 0.05 respect to saline-BDL rats.
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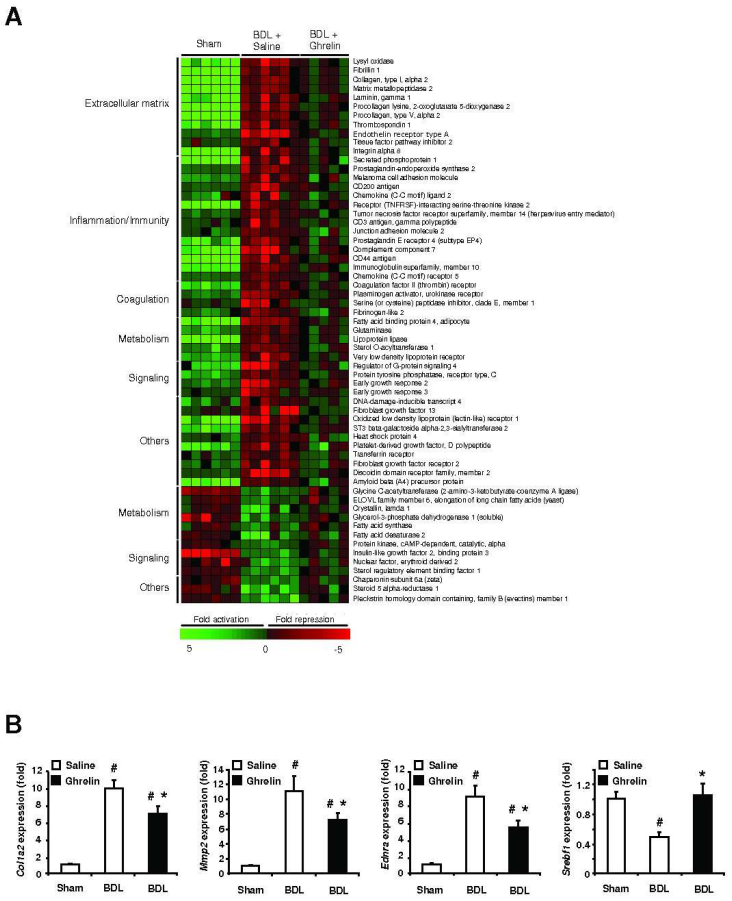


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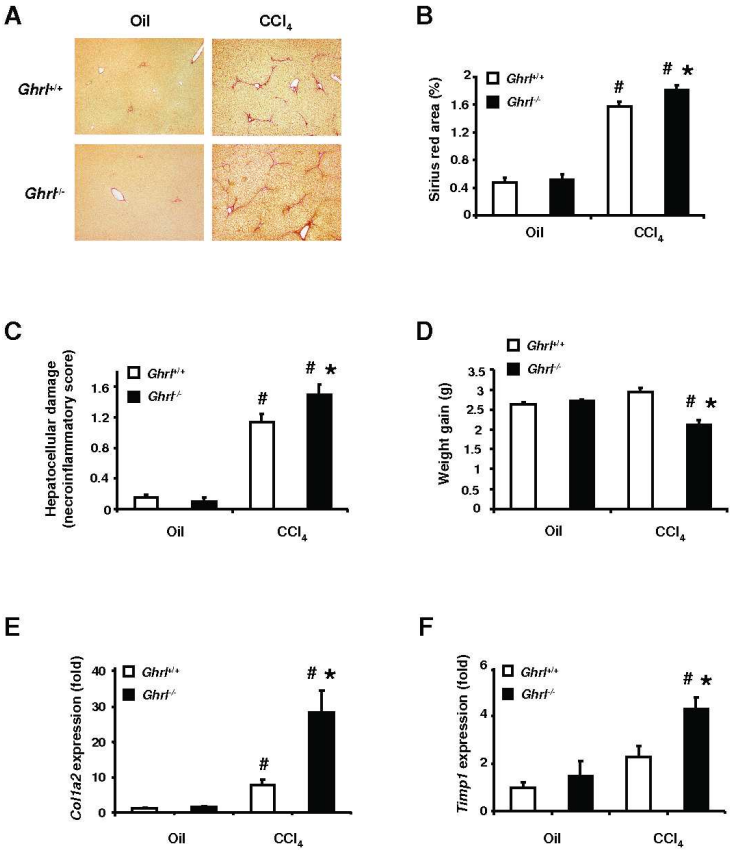


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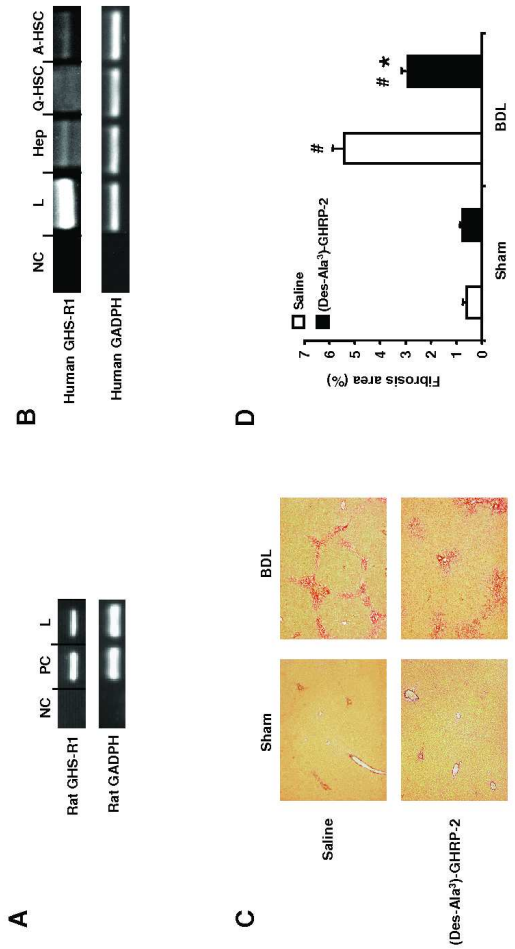


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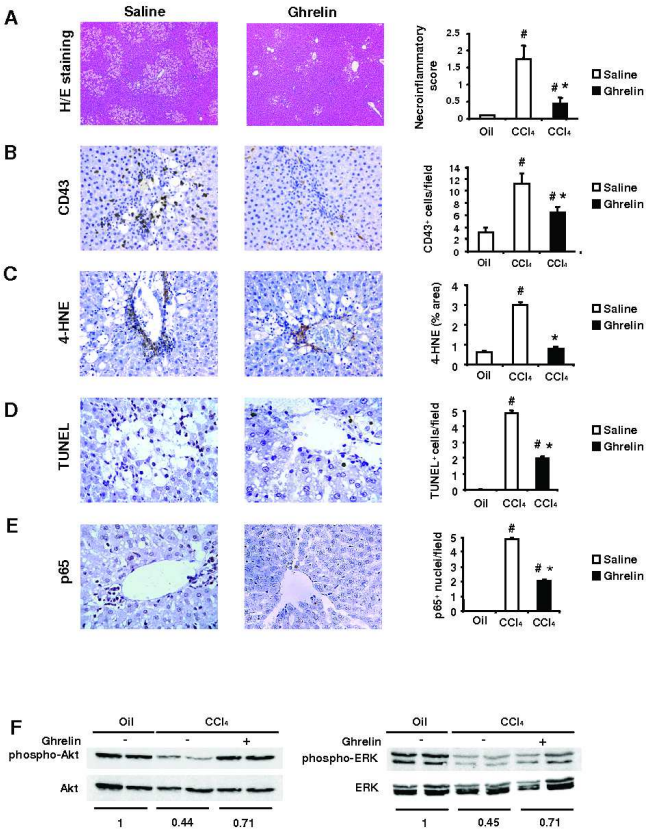


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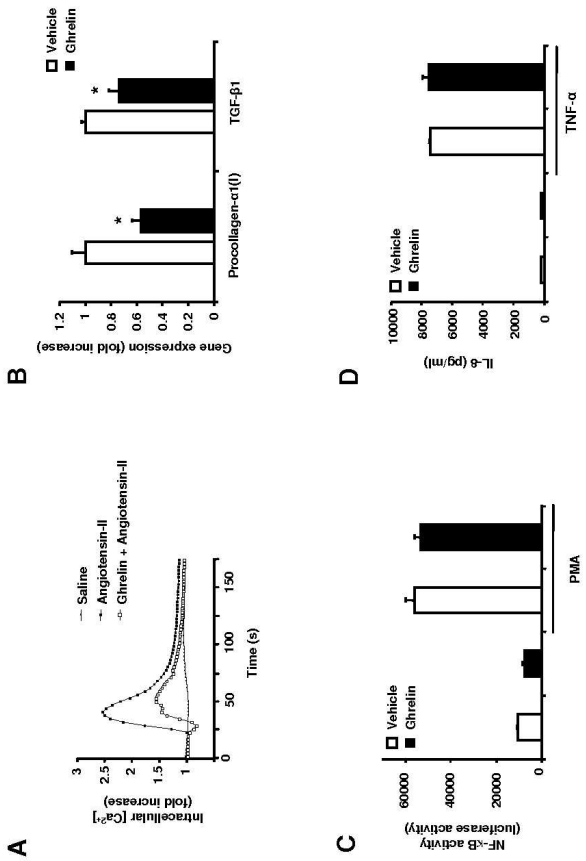


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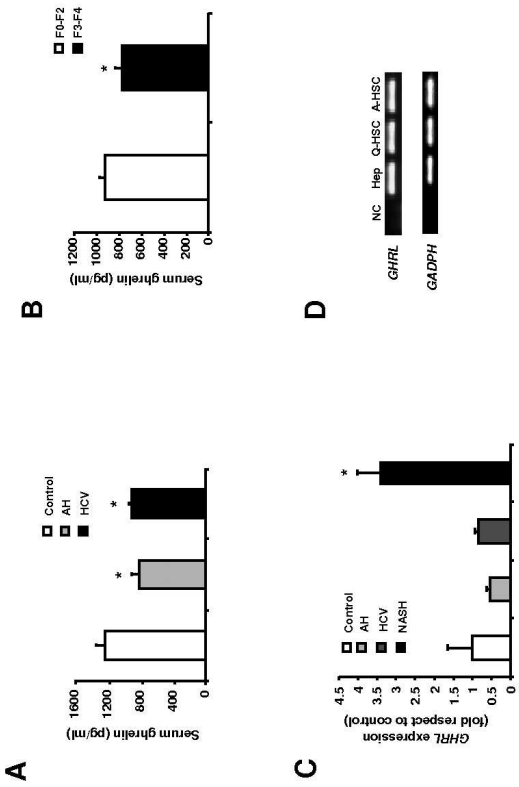


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**Table 1.** Effects of ghrelin genetic polymorphisms in the progress of fibrosis in patients with chronic hepatitis C.

| **-994 CT polymorphism** | | | | | |
|---|---|---|---|---|---|
| **Sex** | **Genotype** | **n (%)** | | **Odd ratio** | **P value** |
| | | **F0-F2** | **F3-F4** | **(95% CI)** | |
| All | CC | 134 (84.3) | 93 (74.4) | 1.00 | -- |
| | TT + CT | 25 (15.7) | 32 (25.6) | 1.79 (0.96-3.37) | .068 |
| Females | CC | 55 (90.2) | 29 (69) | 1.00 | -- |
| | TT+ CT | 6 (9.8) | 13 (31) | **9.75 (1.34-71.05)** | **.010** |
| Males | CC | 79 (80.6) | 64 (77.1) | 1.00 | -- |
| | TT+ CT | 19 (19.4) | 19 (22.9) | 1.01 (0.47-2.19) | .981 |
| **Haplotype** | | | | | |
| **Sex** | **-994 CT** **-604 GA** | **n (%)** | | **Odd ratio** | **P value** |
| | | **F0-F2** | **F3-F4** | **(95% CI)** | |
| All | C A | 82 (51.64) | 63 (50.4) | 1.00 | -- |
| | C G | 64 (40.5) | 44 (35.6) | 0.97 (0.66-1.41) | .850 |
| | T A | 11 (7.17) | 17 (14.0) | **2.06 (1.08-3.91)** | **.028** |
| | T G | 1 (0.7) | 0 (0.0) | 0.00 | 1.00 |
| Females | C A | 32 (51.64) | 21 (51.19) | 1.00 | -- |
| | C G | 26 (43.44) | 13 (30.95) | 0.96 (0.35-2.66) | .943 |
| | T A | 3 (4.92) | 8 (17.86) | **8.47 (1.31-54.84)** | **.029** |
| | T G | 0 (0.00) | 0 (0.00) | 0.00 | 1.00 |
| Males | C A | 51 (51.82) | 42 (50.00) | 1.00 | -- |
| | C G | 38 (38.49) | 31 (37.95) | 1.12 (0.61-2.05) | .712 |
| | T A | 8 (8.39) | 10 (12.05) | 1.40 (0.48-4.05) | .54 |
| | T G | 1 (1.31) | 0 (0.00) | 1.08 (0.00-1088) | .982 |

## SUPPLEMENTARY MATERIALS AND METHODS

### *Cell cultures.*

Human hepatic stellate cells (HSC) were isolated from fragments of normal livers obtained from resections of liver metastasis of colon cancer as described in detail previously.(1) Experiments were performed with HSC activated in culture. Cells were cultured in standard conditions in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) containing 15% fetal bovine serum, non essential aminoacids, glutamine, sodium pyruvate and antibiotics. The protocols were approved by the Investigational Review Board of the Hospital Clínic of Barcelona. Huh7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, glutamine and non essential aminoacids. Cells were serum starved for at least 12 hours before the experiments.

### *Immuhistochemistry studies.*

Paraffine-embedded liver sections were deparaffinized, rehydrated and stained using the DAKO Envision system (DAKO; Carpinteria, CA). To evaluate the degree of liver inflammation, CD43 immunostaining was performed using a monoclonal antibody against CD43 (1:1000, Serotec Inc; Oxford, UK). CD43 positive cells were counted (10 fields per specimen at x200 magnification). p65 immunohistochemistry was performed using a monoclonal antibody against p65 (1:100, Santa Cruz Biotechnology; Santa Cruz, CA). Hepatocytes with p65 positive nuclei per field (30 fields per specimen at x400 magnification) were counted. To evaluate oxidative stress, 4-hydroxynonenal protein adducts (4-HNE) immunostaining was performed (1:200, A.G. Scientific Inc; San Diego, CA). The percentage of positive area (30 fields per specimen at x400 magnification) was calculated as described above for Sirius red. To quantify cell death, Terminal dUTP Nick End Labeling (TUNEL) staining was performed using a commercial kit (Promega Corporation; Madison, WI). Hepatocytes with positive nuclei were counted (10 fields per specimen at x400 magnification). To estimate the amount of fibrogenic myofibroblasts, liver sections were incubated with a monoclonal antibody

against smooth muscle α-actin (α-SMA) (1:1000, DAKO). Positive cells per field were counted (10 fields per specimen at x400 magnification).

***Hepatic hydroxyproline content.***

Hydroxyproline content was quantified colorimetrically from 80 mg of frozen liver samples. Tissue was homogenized in 300 µl of 6N chloridric acid and hydrolyzed at 100°C overnight. The hydrolysate was filtered, aliquots were evaporated under vacuum, and the sediment was redissolved in 50% isopropanol. Samples were then incubated in a solution containing 0.84% chloramine-T 42 mM sodium acetate, 2.6 mM citric acid, and 39.5% (vol/vol) isopropanol (pH 6.0) for 10 minutes at room temperature. Next, samples were incubated in a solution containing 0.248 g *p*-dimethylaminobenzaldehyde dissolved in 0.27 ml of 60% perchloric acid and 0.73 ml of isopropanol for 90 minutes at 50°C. Hydroxyproline content was quantified photometrically at 558 nm. Results are expressed as µmol hydroxyproline per litre of the last solution.

***Gene expression analysis.***

RNA was obtained from cultured cells and human liver samples using the TRIzol reagent (Life Technologies Inc; Rockville, MD). RNA was extracted from rat liver samples by the QIAGEN RNeasy kit. In all cases, retrotranscriptions were performed using the High Capacity cDNA Archive Kit (Applied Biosystems; Foster City, CA). Quantitative PCR were performed using TaqMan gene expression assays (Applied Biosystems) for procollagen-α2(I), metallopeptidase 2, endothelin receptor type A and sterol regulatory element binding factor 1 for rat liver samples, procollagen-α2(I) and tissue inhibitor of metaloproteases (TIMP-1) for mice samples and procollagen-α1(I) and transforming growth factor β for samples from hepatic stellate cells (HSC). TaqMan reactions were carried out in duplicate on an ABI PRISM 7900 Machine (Applied Biosystems). For human liver samples, quantitative PCR for 64 genes were performed using pre-designed TaqMan low density array cards (Applied Biosystems) and carried

out in triplicate on an ABI PRISM 7900HT (Applied Biosystems). All data were normalized to *18S* content and were expressed as fold increase over the control group or as correlations with expression of other genes in case of human liver samples. Reactions for qualitative PCR contained 7.5 ng cDNA , 0.75 µM of each primer, 3.2 µmol/L of each deoxynucleoside triphosphate (dATP, dGTP, dCTP, and dTTP), 1X buffer (HotStar), 0.25X Q solution (Qiagen; Hilden, Germany), and 0.04 $U \cdot \mu L^{-1}$ of DNA polymerase HotStarTaq (Qiagen). Water was added to a final volume of 10 µl. An initial denaturation step at 95℃ for 15 min was followed by 60 cycles of 95℃ for 30 s, annealing temperature for 30 s, and 72℃ for 90 s, with a final step at 72℃ for 15 min. Annealing temperature was 65ºC for the ghrelin gene (*GHRL)* and 58ºC for the growth hormone secretacogue receptor gene (*GHSR).* The size of the PCR products (2-µL aliquot) was analyzed by electrophoresis on 2% agarose gels. The oligonucleotides used for *GHRL* were: 5'-GAGAGTCCAGCAGAGAAAGGAGTC-3' (forward) and 5'-GACAGCTTGATTCCAACATCAAAG-3' (reverse) and the oligonucleotides used for the ghrelin receptor gene (GHSR) were: 5'-CTCTGGACTGCTCACGGTCAT-3' (forward) and 5'- AACACCACTACAGCCAGCATTTT-3' (reverse).

***Microarray studies.***

RNA was isolated from rat livers using the QIAGEN RNeasy kit (Qiagen). RNA integrity was checked with the Agilent 2100 Bioanalyser (Agilent Technologies; Santa Clara, CA) and only high quality RNA samples were hybridized to Rat Genome 230 2.0 GeneChips (Affymetrix; Santa Clara, CA). Briefly, 2 µg of total RNA were used to generate double strand complementary DNA (cDNA) using an oligo dT- primer containing the T7 RNA polymerase promoter site and the SuperScript Choice System kit (Invitrogen; Leek, The Netherlands). cDNA was purified by the GeneChip Sample Clean Up Module, followed by *in vitro* synthesis of biotinylated complementary RNA (cRNA) using the BioArray High Yield RNA transcription kit (Affymetrix). The resulting cRNA was purified and fragmented and 15 µg were hybridized to Rat Genome 230 2.0 GeneChips for 16 hours, at 45ºC and 30 *g*. The arrays were then washed and labelled

with streptavidin-phycoerythrin (SAPE), and the signal was amplified with an anti-streptavidin biotinylated antibody followed by a second round of staining with SAPE using the Affymetrix fluidics station 450. Finally, the labelled arrays were scanned with a Gene chip scanner 3000. Microarray data from 17 samples (6 for the control group, 6 for BDL-saline group, and 5 for the ghrelin-BDL group) were normalized using the guanidine-cytosine content-adjusted robust multiarray algorithm, which computes expression values from probe-intensity values incorporating probe-sequence information. Next, we employed a conservative probe-filtering step excluding those probes not reaching a $\log_2$ expression value of 5 in at least 1 sample, which resulted in the selection of a total of 15,445 probes out of the original 31,099 set. Differential expression was assessed by using linear models and empirical Bayes moderated t-statistics using LIMMA R-package software.(2) Inter-groups comparisons and determinations of false discovery rates (FDR computation using *Benjamini-Hochberg* procedure) for each comparison were performed and FDR values ≤0.2 were deemed potentially significant and selected for further study.

***Assessment of hepatic* guanosin 3',5'-cyclic monophosphate *(cGMP).***

Forty mg of frozen tissue were dropped into 10 volums of 5% trichloroacetic acid (TCA) and homogenized. Precipitated was removed by centrifugation at 1500*g* for 10 min and the supernantant was transferred to a clean test tube. The supernatants were washed with water-saturated diethyl ether three times to remove the TCA. The aqueous suspension and the standars were acetylated and cGMP levels were determined by enzymeimmunoassay (Cayman Chemical Co; Ann Arbor, MI).

***Serum biochemical measurements.***

Serum aspartate aminotransferase (AST) levels were measured using standard enzymatic procedures.

***Measurement of intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$).***

Changes in $[Ca^{2+}]_i$ were measured in Fura-2 (Calbiochem) loaded cells using an inverted epifluorescence microscope as described in detail previously.(3) Cells were pre-incubed for 10 minutes with human recombinant ghrelin (Sigma-Aldrich) or saline and tested with angiotensin-II (Sigma-Aldrich). Cells were considered as responders when $[Ca^{2+}]_i$ increased more than 50% above the resting value.

***NF-κB responsive luciferase assay.***

Human HSC were infected with a recombinant adenoviral vector expressing a luciferase reporter gene driven by nuclear factor κB (NF-κB) transcriptional activation (Ad5NF-κBLuc) for 12 hours. Medium was replaced and cells stimulated with 12-myristate 13-acetate (PMA, Sigma-Adrich) or vehicle for 8 hours. Human recombinant ghrelin or vehicle was added to the medium 10 minutes before PMA. NF-κB-mediated transcriptional induction was assessed by a luciferase assay system kit (Promega Corporation). Cells were serum starved from 12 hours before the adenoviral infection.

***Determination of interleukin 8 secretion.***

Human HSC were cultured in 6-well plates at a density of $4x10^5$ cells/well. Medium was removed and cells incubated in serum-free medium for 24 hours in the presence of tumor necrosis factor α (TNF-α, R&D Systems; Minneapolis, MN) or vehicle. Human recombinant ghrelin was added to the medium 10 minutes before TNF-α. Supernatants were collected and stored at –80ºC until analysis. An enzymeimmunoassay for human interleukin 8 (BLK Diagnostics; Barcelona, Spain) was performed.

***Western blotting.***

Tissue protein extracts were obtained in radio-immunoprecipitation assay (RIPA) lysis buffer containing phosphatase and protease inhibitors. Forty micrograms

of protein were loaded onto 10% sodium dodecyl sulfate-acrylamide gels and blotted onto nitrocellulose membranes. Membranes were then incubated with antibodies against phospho-Akt, Akt, phospho-extracellular signal-regulated kinase (ERK), ERK (Cell Signaling Technology; Berverly, MA), smooth muscle α-actin (DAKO, Carpinteria, CA) or GADPH (Abcam, Cambridge, UK). After extensive washing, membranes were incubated with blocking buffer containing horseradish-peroxidase conjugated secondary antibody. Proteins were detected by chemoluminescence (Amersham Biosciences; Fairfield, CT).

***Serum ghrelin levels analysis.***

Total ghrelin serum levels were analyzed by radioimmunoassay (Linco Research; St. Charles, MI). Blood samples from all subjects were obtained after an overnight fasting in the early morning. Serum was frozen at –80ºC until analysis.

***DNA extraction and genotyping.***

DNA was isolated from peripheral blood cells using the Chemagic System (Chemagen; Baesweiler, Germany). Polymerase chain reaction (PCR) amplicons were designed by Primer3 program(4) to completely traverse the promoter, exon 1, exon 3 and exon 4 of *GHRL*. The size of PCR products was analyzed by electrophoresis on 2% agarose gels. Products were treated with Exonuclease I (Amersham Biosciences) and shrimp alkaline phosphatase (Amersham Biosciences) to remove excess primers and deoxynucleotide triphosphates. For the examination of the six single nucleotide polymorphisms (SNPs), extension SNaPshot primers specific to the polymorphic sites (see table below) were used for the SNapshot minisequencing reaction using the ABI PRISM SNaPshot Multiplex Kit (Applied Biosystems). The resulting products were purified by one unit of Calf Intestine Phosphatase (New England Biolabs, Ipswich, MA). Snapshot products were resuspended in 4,5 µL Hi-Di™ Formamide (Applied Biosystems) and 0.5 µL GeneScan Size Standard. Then, they were electrophoretically

analyzed using a DNA Analyzer 3730 (Applied Biosystems). The results of genotyping were analyzed and evaluated by GeneMapper software v. 3.7 (Applied Biosystems).

***SNPs statistical analysis.***

Allele and genotype frequencies as well as Hardy–Weinberg equilibrium for every SNP were calculated by chi-square test with one degree of freedom. SNPs association with fibrosis were calculated by logistic regression and adjusted by age and gender. Co-dominant, dominant, recessive and over-dominant inheritance models were analyzed for genotype association with fibrosis. The model with lower AIC (Akaike information) was used for every SNP. Haplotypes were stimated by Expectation Maximization algorithm and haplotypes association with fibrosis was calculated by logistic regression models and adjusted by age and gender as covariates. All analysis were performed using SPSS and SNPStats software.(5) We considered statistically significant for association with a *P* value <0.05. Odd ratio was used to evaluate the association of disease state with each SNP or haplotype.

***Construction of the human GHRL promoter-Luciferase plasmids***

The fragment containing 599 bp, corresponding to the -1049 to -450 bp upstream region of the human ghrelin gene (*GHRL*), was amplified by PCR and cloned into pcR2.1-Topo vector (Invitrogen). The (–1049 –450) GHRL-luc plasmid was obtained by releasing restriction fragments from the pcR2.1-Topo constructs followed by subcloning into the pGL3-basic vector (Promega Corporation).

***Lluciferase assay for GHRL promotor activity***

HuH7 cells were seeded in 24-well culture plates and co-transfected using Fugene (Roche applied science, Penzberg, Germany) with 980 ng of GHRL-reporter plasmids and 20 ng of a Renilla luciferase construct (pRL-TK) as an internal control. Transactivation activities were measured 48 h after transfection in a VICTOR3 luminometer (Perkin Elmer, Wellesley, MA) according to the technical manual of the Dual-Luciferase Reporter Assay System (Promega Corporation). The mean firefly

luciferase/renilla ratio was considered. Values represent the means ± S.E.M. of four independent transfection experiments run in duplicate

**Single nucleotide polymorphism information for ghrelin gene (*GHRL*)**

| SNP [A] | Reference [B] | Primers | |
|---|---|---|---|
| **rs26312** | -994 C>T | Forward | TCCTCGGGAAGGTGTAGAATC |
| | | Reverse | AGGCCCAGAGAGGTTAAACG |
| | | -994 C>T* | tagttatatatattaGCTGTTGCTGCTCTGGCCTCT |
| **rs27647** | -604 G>A | Forward | TCCTCGGGAAGGTGTAGAATC |
| | | Reverse | AGGCCCAGAGAGGTTAAACG |
| | | -604 G>A* | atacgttatatTGGGATGGGGTTGCTGGTTTA |
| **rs26802** | –501 A>C | Forward | TCCTCGGGAAGGTGTAGAATC |
| | | Reverse | AGGCCCAGAGAGGTTAAACG |
| | | -501 A>C* | agatatatcgtatgatCAGCAGTCACGGACAATAAACCTG |
| **rs34911341** | Arg51Gln, 304 G>A (exon 3) | Forward | CCTTCCAGCAGAGAAAGGAG |
| | | Reverse | TGTAGTTGGGACCCTGTTCAC |
| | | R51Q* | atatattctatctCGGAGCCAGCCTGCTAGAGCT |
| **rs696217** | Met72Leu, 366 C>A (exon 3) | Forward | CCTTCCAGCAGAGAAAGGAG |
| | | Reverse | TGTAGTTGGGACCCTGTTCAC |
| | | M72T* | cgtcctaGCAGAAGGGGCAGAGGATGAA |
| **rs4684677** | Leu90Gln 421 A>T (exon 4) | Forward | CTGACATCTCCTGGGTCCTC |
| | | Reverse | AAACCGAGCAAACCCAGTC |
| | | L90Q* | taatataactccatattacattaTGGAATCAAGCTGTCAGGGGTTC |

*[A] Single nucleotide polymorphism, [B] Begins in the first nucleotide of first codon of GHRL, accession number of reference sequence was NM_016362 on NCBI.*

** SNaPshot primers for GHRL polymorphism detection.*

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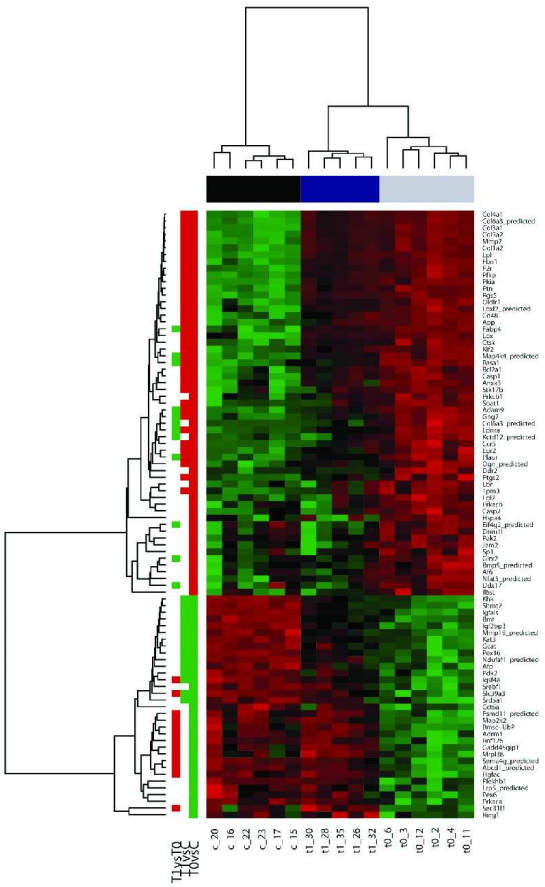


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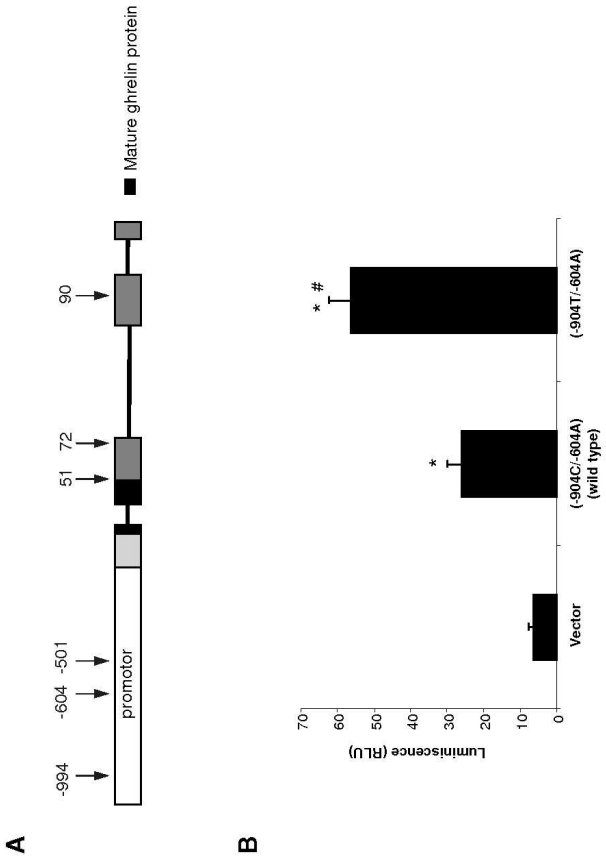


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**Supplemental Table 1.** Effect of ghrelin on hepatic gene expression in rats with fibrosis as assessed by microarray analysis. Only annotated genes were considered.

| Gene symbol | GeneName | BDL-saline vs sham (fold) | BDL-ghrelin vs BDL-saline (fold*) |
|---|---|---|---|
| ***Extracellular matrix*** | | | |
| *Lox* | lysyl oxidase | 41.97 | -2.61 |
| *Plaur* | plasminogen activator, urokinase receptor | 4.17 | -2.50 |
| *Sparcl1* | SPARC-like 1 (mast9, hevin) | 5.77 | -2.50 |
| *Cthrc1* | collagen triple helix repeat containing 1 | 8.17 | -2.41 |
| *Plod2* | procollagen lisine, 2-oxoglutarate 5-dioxygenase 2 | 25.64 | -2.19 |
| *Lamc1* | laminin, gamma 1 | 10.48 | -1.97 |
| *Mmp2* | matrix metallopeptidase 2 | 15.42 | -1.95 |
| *Fbn1* | fibrillin 1 | 11.87 | -1.93 |
| *Fgl2* | fibrinogen-like 2 | 2.46 | -1.92 |
| *Adam9* | a disintegrin and metalloproteinase domain 9 (meltrin gamma) | 1.58 | -1.89 |
| *Timp3* | tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory) | 1.66 | -1.84 |
| *Mgp* | matrix Gla proteín | 18.36 | -1.81 |
| *Mxra8* | matrix-remodelling associated 8 | 5.31 | -1.77 |
| *Ermp1* | endoplasmic reticulum metallopeptidase 1 | 1.92 | -1.76 |
| *Plat* | plasminogen activator, tissue | 16.99 | -1.75 |
| *Thbs1* | thrombospondin 1 | 17.34 | -1.74 |
| *Col5a2* | procollagen, type V, alpha 2 | 11.51 | -1.72 |
| *Col1a1* | procollagen, type 1, alpha 1 | 24.85 | -1.67 |
| *Loxl1* | lysyl oxidase-like 1 | 118.96 | -1.65 |
| *Col4a1* | procollagen, type IV, alpha 1 | 13.60 | -1.64 |
| *Col12a1* | procollagen, type XII, alpha 1 | 12.64 | -1.63 |
| *Col3a1* | procollagen, type III, alpha 1 | 5.64 | -1.60 |
| *Ltbp1* | latent transforming growth factor beta binding protein 1 | 8.72 | -1.59 |

| | | | |
|---|---|---|---|
| *Col5a1* | procollagen, type V, alpha 1 | 5.43 | -1.53 |
| *Reln* | reelin | 1.60 | -1.50 |
| ***Inflammation / Immunity*** | | | |
| *C7* | complement component 7 | 20.03 | -2.85 |
| *Colec12* | collectin sub-family member 12 | 12.89 | -2.49 |
| *Spp1* | secreted phosphoprotein 1 | 46.04 | -2.38 |
| *Mcam* | melanoma cell adhesion molecule | 4.45 | -2.34 |
| *Tnfrsf14* | tumor necrosis factor receptor superfamily, member 14 (herpesvirus entry mediator) | 2.95 | -2.34 |
| *Cd200* | Cd200 antigen | 3.15 | -2.19 |
| *Ahr* | aryl hydrocarbon receptor | 1.71 | -2.16 |
| *Cd3g* | CD3 antigen, gamma polypeptide | 2.15 | -1.89 |
| *Igsf10* | immunoglobulin superfamily, member 10 | 7.58 | -1.83 |
| *Ccl2* | chemokine (C-C motif) ligand 2 | 17.57 | -1.83 |
| *Cd44* | CD44 antigen | 54.65 | -1.78 |
| *Tnfrsf11b* | tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin) | 2.78 | -1.70 |
| *Catna1* | catenin (cadherin-associated protein), alpha 1 | 1.83 | -1.67 |
| *Cd38* | CD38 antigen | 2.19 | -1.64 |
| *Ccr5* | chemokine (C-C motif) receptor 5 | 2.32 | -1.63 |
| *Af6* | Afadin | 1.76 | -1.58 |
| *Irf8* | interferon regulatory factor 8 | 2.10 | -1.57 |
| *Tia1* | cytotoxic granule-associated RNA binding protein 1 | 2.06 | -1.55 |
| *Sla* | src-like adaptor | 2.79 | -1.54 |
| *Cd276* | CD276 antigen | 6.50 | -1.50 |
| *Igha_mapped* | immunoglobulin heavy chain (alpha polypeptide) (mapped) | -4.17 | 2.73 |
| ***Energetic metabolism*** | | | |
| *Oldlr1* | oxidized low density lipoprotein (lectin-like) receptor 1 | 16.44 | -2.87 |
| *Fabp4* | fatty acid binding protein 4, adipocyte | 23.44 | -2.55 |
| *Vldlr* | very low density lipoprotein receptor | 5.29 | -2.11 |
| *B3galt3* | UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, | 17.61 | -2.07 |

| | | | |
|---|---|---|---|
| | polypeptide 3 | | |
| *Mlstd2* | male sterility domain containing 2 | 2.94 | -2.07 |
| *Lpl* | lipoprotein lipase | 25.40 | -2.01 |
| *St3gal2* | ST3 beta-galactoside alpha-2,3-sialyltransferase 2 | 6.55 | -1.83 |
| *Pfkp* | Phosphofructokinase, platelet | 5.18 | -1.70 |
| *Soat1* | sterol O-acyltransferase 1 | 3.72 | -1.64 |
| *Pdk3* | pyruvate dehydrogenase kinase, isoenzyme 3 | 6.33 | -1.57 |
| *St3gal4* | ST3 beta-galactoside alpha-2,3-sialyltransferase 4 | 3.40 | -1.55 |
| *Asah1* | N-acylsphingosine amidohydrolase 1 | 3.33 | -1.50 |
| *Acly* | ATP citrate lyase | -2.88 | 1.50 |
| *Ptms* | Parathymosin | -1.99 | 1.50 |
| *Dhcr7* | 7-dehydrocholesterol reductase | -1.85 | 1.52 |
| *Igfals* | insulin-like growth factor binding protein, acid labile subunit | -3.47 | 1.56 |
| *Dcxr* | dicarbonyl L-xylulose reductase | -3.61 | 1.60 |
| *Pdk2* | pyruvate dehydrogenase kinase, isoenzyme 2 | -2.45 | 1.61 |
| *Igf2bp3* | insulin-like growth factor 2, binding protein 3 | -5.19 | 1.79 |
| *Fasn* | fatty acid synthase | -1.87 | 1.87 |
| *Elovl6* | ELOVL family member 6, elongation of long chain fatty acids (yeast) | -2.23 | 1.88 |
| *Gcat* | glycine C-acetyltransferase (2-amino-3-ketobutyrate-coenzyme A ligase) | -4.28 | 1.97 |
| *Gpd1* | glycerol-3-phosphate dehydrogenase 1 (soluble) | -4.26 | 2.00 |
| *Cryl1* | Crystallin, lamda 1 | -2.44 | 2.09 |
| *Aacs* | acetoacetyl-CoA synthetase | -1.59 | 2.21 |
| *Fads2* | fatty acid desaturase 2 | -3.10 | 2.24 |
| ***Metabolism*** | | | |
| *Cyp1b1* | cytochrome P450, family 1, subfamily b, polypeptide 1 | 2.37 | -2.18 |
| *Cybb* | cytochrome b-245, beta polypeptide | 4.30 | -2.06 |
| *Heph* | Hephaestin | 6.66 | -1.95 |
| *Gls* | Glutaminase | 3.85 | -1.83 |
| *Cybrd1* | cytochrome b reductase 1 | 1.84 | -1.74 |

| | | | |
|---|---|---|---|
| *Glrx2* | glutaredoxin 2 (thioltransferase) | 1.91 | -1.70 |
| *Hprt* | hypoxanthine guanine phosphoribosyl transferase | 1.84 | -1.64 |
| *Chdh* | choline dehydrogenase | -3.40 | 1.50 |
| *Hfe2* | hemochromatosis type 2 (juvenile) homolog (human) | -1.60 | 1.59 |
| *Cyp2t1* | cytochrome P450 monooxygenase CYP2T1 | -3.69 | 1.62 |
| *Dao1* | D-amino acid oxidase 1 | -3.99 | 1.64 |
| *Prodh2* | proline dehydrogenase (oxidase) 2 | -2.67 | 1.70 |
| *Sts* | steroid sulfatase | -2.27 | 1.75 |
| *Abat* | 4-aminobutyrate aminotransferase | -6.01 | 1.77 |
| *Gstm2* | glutathione S-transferase, mu 2 | -3.46 | 1.77 |
| *Srd5a1* | steroid 5 alpha-reductase 1 | -4.03 | 2.29 |
| ***Signaling*** | | | |
| *Rgs4* | regulator of G-protein signaling 4 | 10.05 | -4.29 |
| *Prkacb* | protein kinase, cAMP dependent, catalytic, beta | 1.55 | -2.73 |
| *Egr2* | early growth response 2 | 4.92 | -2.51 |
| *Zfhx1b* | zinc finger homeobox 1b | 1.99 | -2.26 |
| *Arl11* | ADP-ribosylation factor-like 11 | 6.01 | -2.25 |
| *Pkia* | protein kinase inhibitor, alpha | 12.72 | -2.04 |
| *Ptprc* | protein tyrosine phosphatise, receptor type, C | 4.08 | -1.99 |
| *Gng2* | guanine nucleotide binding proteína, gamma 2 | 2.76 | -1.97 |
| *Egr3* | early growth response 3 | 2.07 | -1.97 |
| *Gadd45b* | growth arrest and DNA-damage-inducible 45 beta | 1.93 | -1.94 |
| *Sp1* | Sp1 transcription factor | 1.70 | -1.87 |
| *Edg2* | endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2 | 3.44 | -1.85 |
| *Ddit4* | DNA-damage-inducible transcript 4 | 2.84 | -1.84 |
| *Slk* | serine/threonine kinase 2 | 1.75 | -1.80 |
| *Bhlhb3* | basic helix-loop-helix domain containing, class B3 | 2.06 | -1.74 |
| *Adcy3* | adenylate cyclase 3 | 1.96 | -1.73 |
| *Plcl1* | phospholipase C-like 1 | 3.04 | -1.73 |
| *Prkcb1* | protein kinase C, beta 1 | 2.71 | -1.70 |

| | | | |
|---|---|---|---|
| *Gucy1a3* | guanylate cyclase 1, soluble, alpha 3 | 1.86 | -1.67 |
| *Anxa3* | annexin A3 | 2.82 | -1.67 |
| *Rem1* | rad and gem related GTP binding protein 1 | 1.78 | -1.65 |
| *Pld1* | phospholipase D1 | 2.31 | -1.63 |
| *Rgs5* | regulator of G-protein signaling 5 | 8.51 | -1.62 |
| *Prkaa1* | protein kinase, AMP-activated, alpha 1 catalytic subunit | 1.88 | -1.61 |
| *Tcf21* | transcription factor 21 | 4.61 | -1.60 |
| *Tfec* | transcription factor EC | 3.08 | -1.58 |
| *Mtf2* | metal response element binding TF 2 | 1.58 | -1.58 |
| *Ptprz1* | protein tyrosine phosphatise, receptor-type, Z polypeptide 1 | 9.63 | -1.57 |
| *Rgs2* | regulator of G-protein signaling 2 | 10.46 | -1.57 |
| *Stk17b* | serine/threonine kinase 17b (apoptosis-inducing) | 2.79 | -1.56 |
| *Arpp19* | cAMP-regulated phosphoprotein 19 | 2.91 | -1.56 |
| *Hnrpa3* | heterogeneous nuclear ribonucleoprotein A3 | 1.57 | -1.56 |
| *Ap2b1* | adaptor-related protein complex 2, beta 1 subunit | 2.00 | -1.55 |
| *Znf292* | zinc finger protein 292 | 1.60 | -1.54 |
| *Akap13* | A kinase (PRKA) anchor protein 13 | 3.21 | -1.54 |
| *Runx3* | runt-related transcription factor 3 | 1.90 | -1.54 |
| *Atm* | ataxia telangiectasia mutated homolog (human) | 1.63 | -1.54 |
| *Pak2* | p21 (CDKN1A)-activated kinase 2 | 1.61 | -1.52 |
| *Prkch* | protein kinase C, eta | 1.90 | -1.52 |
| *Rhoq* | ras homolog gene family, member Q | 2.53 | -1.51 |
| *Dab2* | disabled homolog 2 (Drosophila) | 3.05 | -1.51 |
| *Anxa2* | annexin A2 | 15.79 | -1.50 |
| *Rnf39* | ring finger protein 39 | -2.67 | 1.99 |
| *Nfe2* | nuclear factor, erythroid derived 2 | -3.17 | 1.89 |
| *Prkaca* | protein kinase, cAMP-dependent, catalytic, alpha | -1.95 | 1.78 |
| *Hes6* | hairy and enhancer of split 6 (Drosophila) | -3.68 | 1.71 |
| *Rnf126* | ring finger protein 126 | -1.67 | 1.70 |
| *Srebf1* | sterol regulatory element binding factor 1 | -2.95 | 1.67 |
| *Rgs3* | regulator of G-protein signalling 3 | -2.55 | 1.58 |

| | | | |
|---|---|---|---|
| *Tcf1* | transcription factor 1 | -1.72 | 1.57 |
| ***Membrane proteins*** | | | |
| *Jam2* | junction adhesion molecule 2 | 2.17 | -2.29 |
| *Abcc5* | ATP-binding cassette, sub-family C (CFTR/MRP), member 5 | 2.65 | -2.06 |
| *Itga8* | integrin alpha 8 | 14.03 | -1.96 |
| *Gja1* | gap junction membrane channel protein alpha 1 | 12.37 | -1.87 |
| *Slc25a4* | solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4 | 8.49 | -1.68 |
| *Slc39a6* | solute carrier family 39 (metal ion transporter), member 6 | 2.06 | -1.58 |
| *Gja7* | gap junction membrane channel protein alpha 7 | 3.18 | -1.51 |
| *Slc17a5* | solute carrier family 17 (anion/sugar transporter), member 5 | -1.79 | 1.51 |
| *Atp6v0a1* | ATPase, H+ transporting, lysosomal V0 subunit A1 | -1.55 | 1.57 |
| *Slc39a3* | solute carrier family 39 (zinc transporter), member 3 | -2.10 | 1.57 |
| *Abcd3* | ATP-binding cassette, sub-family D (ALD), member 3 | -2.02 | 1.58 |
| *Slc26a1* | solute carrier family 26 (sulfate transporter), member 1 | -3.12 | 1.62 |
| *Slc23a1* | solute carrier family 23 (nucleobase transporters), member 1 | -4.44 | 1.64 |
| *Adrm1* | adhesion regulating molecule 1 | -1.81 | 1.90 |
| ***Vasoactive substances/Coagulation*** | | | |
| *Ddr2* | discoidin domain receptor family, member 2 | 3.50 | -3.06 |
| *Serpine1* | serine (or cysteine) peptidase inhibitor, clade E, member 1 | 2.36 | -2.33 |
| *Ednra* | endothelin receptor type A | 2.54 | -1.93 |
| *Tfpi2* | tissue factor pathway inhibitor 2 | 1.82 | -1.86 |
| *F2r* | coagulation factor II (thrombin) receptor | 6.01 | -1.82 |
| *Ednrb* | endothelin receptor type B | 9.69 | -1.80 |
| *Ptafr* | platelet-activating factor receptor | 2.15 | -1.67 |
| *Adra1b* | adrenergic receptor, alpha 1b | -3.60 | 1.56 |
| ***Apoptosis*** | | | |
| *Ripk2* | receptor (TNFRSF)-interacting serine-threonine kinase 2 | 2.55 | -1.88 |
| *Bcl2a1* | B-cell leukemia/lymphoma 2 related protein A1 | 4.99 | -1.80 |
| *Casp2* | caspase 2 | 2.09 | -1.77 |
| *Casp1* | caspase 1 | 3.60 | -1.68 |

| | | | |
|---|---|---|---|
| *Bmf* | Bcl2 modifying factor | -7.09 | 1.72 |
| ***Cytoskeleton*** | | | |
| *Eml2* | echinoderm microtubule associated protein like 2 | 3.01 | -2.02 |
| *Tpm4* | tropomyosin 4 | 7.64 | -1.68 |
| *Lbr* | lamin B receptor | 2.05 | -1.67 |
| *Kif2* | kinesin heavy chain family, member 2 | 3.26 | -1.57 |
| *Tpm3* | tropomyosin 3, gamma | 2.09 | -1.55 |
| ***Growth factors*** | | | |
| *Fgf13* | fibroblast growth factor 13 | 3.93 | -4.03 |
| *Pdgfd* | platelet-derived growth factor, D polypeptide | 7.09 | -2.67 |
| *Fgfr2* | fibroblast growth factor receptor 2 | 3.26 | -2.25 |
| *Ptn* | Pleiotrophin | 6.41 | -1.71 |
| *Fgfr1* | Fibroblast growth factor receptor 1 | 5.68 | -1.68 |
| *Hgf* | hepatocyte growth factor | 2.67 | -1.56 |
| *Pdgfra* | platelet derived growth factor receptor, alpha polypeptide | 4.16 | -1.51 |
| ***Others*** | | | |
| *Emp1* | epithelial membrane protein 1 | 6.36 | -3.05 |
| *RT1-Aw2* | RT1 class Ib, locus Aw2 | 2.54 | -2.75 |
| *Sf3b1* | splicing factor 3b, subunit 1 | 1.56 | -2.66 |
| *Cdh11* | cadherin 11 | 10.61 | -2.53 |
| *Nedd4* | neural precursor cell expressed, developmentally down-regulated gene 4 | 2.09 | -2.39 |
| *Ogt* | O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase) | 1.77 | -2.15 |
| *Hspa4* | heat shock protein 4 | 2.24 | -2.02 |
| *Kitl* | kit ligand | 4.91 | -1.99 |
| *Crygc* | Crystallin, gamma C | 2.15 | -1.92 |
| *Fblim1* | filamin binding LIM protein 1 | 15.74 | -1.92 |
| *Olfml1* | olfactomedin-like 1 | 3.18 | -1.86 |

| | | | |
|---|---|---|---|
| *Pqlc3* | PQ loop repeat containing 3 | 27.64 | -1.84 |
| *Tfrc* | Transferrin receptor | 2.13 | -1.80 |
| *Fstl1* | follistatin-like 1 | 4.95 | -1.78 |
| *Ctsk* | Cathepsin K | 3.98 | -1.78 |
| *Spnb2* | spectrin beta 2 | 3.64 | -1.75 |
| *Cugbp2* | CUG triplet repeat, RNA binding protein 2 | 2.68 | -1.73 |
| *Osbpl5* | oxysterol binding protein-like 5 | 4.03 | -1.72 |
| *Pcsk1* | Proprotein convertase subtilisin/kexin type 1 | 4.81 | -1.70 |
| *Clecsf6* | C-type (calcium dependent, carbohydrate recognition domain) lectin, superfamily member 6 | 5.00 | -1.70 |
| *Ssg1* | steroid sensitive gene 1 | 4.68 | -1.69 |
| *Fhl2* | four and a half LIM domains 2 | 25.51 | -1.69 |
| *Ddx46* | DEAD (Asp-Glu-Ala-Asp) box polypeptide 46 | 2.49 | -1.69 |
| *Ppic* | peptidylprolyl isomerase C | 26.91 | -1.68 |
| *Ctse* | Cathepsin E | 5.74 | -1.68 |
| *RT1-Ba* | RT1 class II, locus Ba | 3.04 | -1.65 |
| *Mgl1* | Macrophage galactose N-acetyl-galactosamine specific lectin 1 | 3.26 | -1.65 |
| *Robo2* | roundabout homolog 2 (Drosophila) | 12.90 | -1.64 |
| *Sfpq* | splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated) | 1.53 | -1.63 |
| *Ddx17* | DEAD (Asp-Glu-Ala-Asp) box polypeptide 17 | 1.58 | -1.61 |
| *Ddx21a* | DEAD (Asp-Glu-Ala-Asp) box polypeptide 21a | 1.55 | -1.61 |
| *Lgals1* | Lectin, galactose binding, soluble 1 | 18.79 | -1.60 |
| *Gpiap1* | GPI-anchored membrane protein 1 | 1.90 | -1.59 |
| *RT1-N3* | RT1 class Ib gene, H2-TL-like, grc region (N3) | 1.95 | -1.58 |
| *S100a6* | S100 calcium binding protein A6 (calcyclin) | 30.28 | -1.55 |
| *App* | amyloid beta (A4) precursor protein | 6.69 | -1.55 |
| *Mdn1* | midasin homolog (yeast) | 1.53 | -1.54 |
| *Cdr2* | cerebellar degeneration-related 2 | 1.97 | -1.54 |
| *Rab31* | RAB31, member RAS oncogene family | 5.25 | -1.54 |
| *RT1-Da* | RT1 class II, locus Da | 4.03 | -1.53 |

| | | | |
|---|---|---|---|
| *Trip10* | thyroid hormone receptor interactor 10 | 1.65 | -1.53 |
| *Btg3* | B-cell translocation gene 3 | 7.20 | -1.51 |
| *Plekhb1* | pleckstrin homology domain containing, family B (evectins) member 1 | -3.18 | 2.33 |
| *Cct6a* | chaperonin subunit 6a (zeta) | -2.53 | 1.81 |
| *Ddhd1* | DDHD domain containing 1 | -2.65 | 1.78 |
| *Bmsc-UbP* | bone marrow stromal cell-derived ubiquitin-like protein | -1.57 | 1.76 |
| *Cml4* | camello-like 4 | -48.15 | 1.74 |
| *Npy* | neuropeptide Y | -1.75 | 1.72 |
| *Xkr8* | X Kell blood group precursor related family member 8 homolog | -2.71 | 1.68 |
| *Mig12* | MID1 interacting G12-like proteína | -1.98 | 1.66 |
| *Snrpn* | small nuclear ribonucleoprotein N | -1.53 | 1.63 |
| *Pex16* | peroxisome biogenesis factor 16 | -2.82 | 1.54 |
| *Kat3* | kynurenine aminotransferase III | -2.90 | 1.52 |
| *Lrp16* | LRP16 protein | -3.09 | 1.51 |

*At least 50% of variation respect to BDL-saline. FDR < 0.2

**Supplemental Table 3.** Correlation of *GHRL* hepatic expression with expression of other genes in patients with chronic liver diseases.

| Gene Symbol | Gene name | Group | r | P value |
|---|---|---|---|---|
| *SERPINE1* | Plasminogen activator inhibitor type 1 | A | 0.713 | <0.0001 |
| *TGFB1* | Transforming growth factor beta 1 | A | 0.708 | <0.0001 |
| *ACE* | Angiotensin I converting enzyme | A | 0.641 | <0.0001 |
| *TNFRSF1B* | Tumor necrosis factor receptor superfamily, member 1B | A | 0.687 | <0.0001 |
| *ADIPOR1* | Adiponectin receptor 1 | B | 0.671 | <0.0001 |
| *IGF1* | Insulin-like growth factor 1 | B | 0.624 | <0.0001 |
| *IRS1* | Insulin receptor substrate 1 | B | 0.642 | <0.0001 |
| *PBEF1* | Visfatin | B | 0.673 | <0.0001 |
| *ABCG1* | ATP-binding cassette, sub-family G member 1 | C | 0.703 | <0.0001 |
| *ABCG8* | ATP-binding cassette, sub-family G member 8 | C | 0.671 | <0.0001 |
| *ABCG5* | ATP-binding cassette, sub-family G member 5 | C | 0.690 | <0.0001 |
| *SP2* | Sp2 transcription factor | D | 0.642 | <0.0001 |
| *JAK1* | Janus kinase 1 | D | 0.694 | <0.0001 |
| *SREBF1* | Sterol regulatory element binding transcription factor 1 | D | 0.702 | <0.0001 |
| *SOCS1* | Suppressor of cytokine signaling 1 | D | 0.628 | <0.0001 |
| *STAT3* | Signal transducer and activator of transcription 3 | D | 0.688 | <0.0001 |
| *SP1* | Sp1 transcription factor | D | 0.671 | <0.0001 |
| *JAK2* | Janus kinase 2 | D | 0.705 | <0.0001 |
| *PPARG* | Peroxisome proliferator-activated receptor gamma | D | 0.701 | <0.0001 |
| *PPARD* | Peroxisome proliferator-activated receptor delta | D | 0.665 | <0.0001 |
| *PPARA* | Peroxisome proliferator-activated receptor alpha | D | 0.631 | <0.0001 |
| *SREBF2* | Sterol regulatory element binding transcription factor 2 | D | 0.666 | <0.0001 |
| *ATF4* | Activating transcription factor 4 | D | 0.673 | <0.0001 |
| *EIF2AK3* | Eukaryotic translation initiation factor 2-alpha kinase 3 | E | 0.647 | <0.0001 |
| *HMGCR* | 3-hydroxy-3-methylglutaryl-Coenzyme A reductase | E | 0.646 | <0.0001 |
| *SMPD1* | Sphingomyelin phosphodiesterase 1, acid lysosomal | E | 0.699 | <0.0001 |
| *HSP5A* | Heat shock protein 5 | E | 0.668 | <0.0001 |

A. Fibrosis/inflammation, B. Hormones/adipokines, C. Transporters, D. Intracellular signaling, E. Others

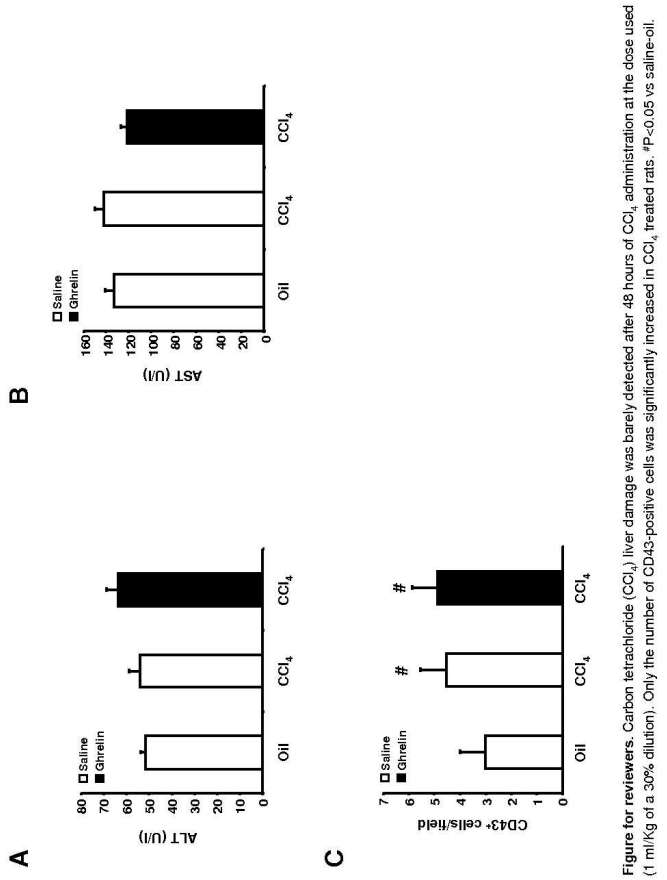


**Figure for reviewers.** Carbon tetrachloride ($CCl_4$) liver damage was barely detected after 48 hours of $CCl_4$ administration at the dose used (1 ml/Kg of a 30% dilution). Only the number of CD43-positive cells was significantly increased in $CCl_4$ treated rats. #P<0.05 vs saline-oil.

209x297mm (150 x 150 DPI)